Lasers in Surgery and Medicine 20:451–460 (1997)

Helium-Neon Laser Irradiation Is Not a Stressful Treatment: A Study on Heat-Shock Protein (HSP70) Level

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Background and Objective: **Helium-neon (He-Ne) laser irradiation has been clinically used to reduce chemotherapy-induced mucositis. This work was designed to find out if this treatment is stressful at the cellular level by studying its effects on the level of the stress-inducible heat shock proteins.**
Study Design/Materials and Methods: **Human desmodontal and mouse L929 fibroblasts were irradiated using a 60 mW laser by a single application of 1.5 and 3J/cm^2 in continuous mode. Heat shock protein level was studied by gel electrophoresis and Western blotting using monoclonal antibodies.**
Results: **He-Ne laser treatment does not induce heat shock protein synthesis in human desmodontal nor in mouse fibroblasts at the energy densities used in this study.**
Conclusion: **These results indicate that the treatment is not stressful at the cellular level. Lasers Surg. Med. 20:451–460, 1997.**

Key words: desmodontal fibroblasts; heat-shock protein (HSP70); He-Ne laser irradiation; mucositis; stressful treatment

INTRODUCTION

Cytotoxic cancer drug treatment results in the development of oral mucositis in 36–100% of patients [1, 2]. The clinical manifestation consists of erythema, edema, and progresses to ulceration with or without pseudomembrane formation. Mucosal bleeding also may occur with thrombocytopenia and oral ulceration. Lesions are accompanied by symptoms ranging from mild burning to severe pain [3–6]. These clinical changes have important systemic implications for myelosuppressed bone marrow transplantation (BMT) patients, including risk of microbial invasion with regional or systemic infections. In addition, mastication and deglutition may be intolerable requiring parenteral nutrition [5, 7, 8].

The prevention and treatment of chemotherapy-induced mucositis and infection have not been uniformly successful. Reduction in the severity or duration of mucositis by chlorhexidine and other mouth rinses has been reported for cancer patients [9, 14]. Oral cryotherapy was relatively successful in inhibiting 5-Fluorouracil-induced stomatitis [15]. Helium-neon (He-Ne) laser irradiation is another approach that proved beneficial in the prevention and treatment of mucositis. Recent studies have shown that He-Ne laser treatment reduces the incidence and the severity of chemotherapy-induced oral mucositis in BMT patients [16, 17].

Although some investigators have reported negative results, such as the absence of effect of He-Ne laser irradiation on wound healing [18, 19], many other beneficial effects have been noted. For example, He-Ne laser irradiation has been shown to induce pain relief [20, 21], and it has been used in the therapy of peptic ulcers and arthritis [22–24]. Experimental studies in animals and clinical observations provide evidence that He-Ne laser treatment of cutaneous and periodontal lesions accelerates the process of wound

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Accepted for publication 25 July 1996.

healing [21, 25]. Laser irradiation of open wounds also is claimed to stimulate fibroblast replication [26]. It has been suggested that low-energy radiation stimulates skin regeneration by inducing mitotic activity of epithelial cells [21] and increasing collagen synthesis through selective activation of collagen gene expression [27].

In spite of the large body of evidence of the beneficial role He-Ne laser irradiations in clinic, very little is known about the side effects of such treatments at the cellular level. Although clinical studies showed no significant side effects of He-Ne irradiations [22, 28], He-Ne laser irradiation has been shown to induce significant ultrastructural changes of epithelial cells in vitro [29, 30] and modify enzyme properties [31]. But the cellular mechanisms of the effects of He-Ne irradiation are poorly understood. They seem to be due to biochemical rather than thermal effects [29]. However, even if no rise in temperature was observed after He-Ne laser irradiation, the treatment may be stressful (i.e., stimulates the synthesis of stress proteins indicating an alteration of the metabolic pathways due to an oxidative stress leading to free radical formation and proteins missfolding and aggregation). In order to find out if the laser irradiation is a stressful treatment, the induction of stress protein synthesis provides a useful means.

When the cells or organisms are exposed to adverse conditions such as heat and metabolic poisons, they respond by synthesizing a characteristic group of proteins termed “heat-shock proteins” (HSP) [32]. Recent work has demonstrated that the synthesis of heat-shock protein is regulated by heat-shock transcription factors, which, when activated in response to a stress, bind to heat-shock elements and regulate gene expression [33–35]. Heat-shock transcription factors have been shown to be responsive to both heat and radiation stress [33, 35], although it is possible that different factors are involved. Even though the biological significance of HSP is still being defined, one of the proposed functions of HSP is the protection of cells against a wide variety of environmental stresses. It has been reported that nearly all species synthesize HSP belonging to three different gene families, with molecular masses in the range of 80–90, 68–75, and 15–30 KDa, respectively [36]. Within these HSP families, the HSP70 family is the most conserved and the best characterized [32, 36, 37].

In mammalian cells, there are two prominent forms of the HSP70 family, an abundant constitution protein, and a highly stress-induced one [38–40]. Under normal growth conditions, members of the HSP70 family have been implicated in the stabilization of unfolded protein precursors before assembly in the cytosol [38], or translocation into organelles including the endoplasmic reticulum and mitochondria [41, 42], in stabilization of newly translocated polypeptides before folding and assembly [43–45], in dissolution of protein aggregates [45], and in the degradation of rapidly turned over cytosolic proteins [46].

An increased expression of HSP70 in cells undergoing stress seems to be needed to protect these metabolic pathways and to reduce cell damage. Upon stress, HSP70 has been shown to reactivate unfolded and aggregated proteins [36] and accelerate the degradation of proteins denatured beyond repair [46]. HSP70 are expressed at low levels under normal conditions, but they are highly induced by heat shock or many other stressful agents such as heavy metals amino acid analogues, ethanol, oxidants, and toxins [47]. Recently, it has been shown that ultraviolet irradiation (280–320 nm and 254 nm) of organ cultured normal human skin induce HSP70 protein synthesis [48].

This work was designed to find out if He-Ne laser irradiation is stressful to cells directly subjected to this treatment by studying its effects on heat-shock protein (HSP70) synthesis.

MATERIALS AND METHODS

Human desmodontal fibroblasts and mouse L929 cell line were used to find out if He-Ne laser irradiation is stressful at the cellular level by studying the effect of this treatment on the induction of stress proteins in these cell types.

Alkaline phosphatase-conjugated antimouse IgG (whole molecule), bovine serum albumin, Tween 20, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, Coomassie Blue R250 were purchased from Sigma (Sigma Chemical Co., St Louis, MO). Nitrocellulose membranes were from Schleicher and Schuell (Dassel, Germany), and phenylmethylsulfonyl fluoride, benzamidine from Fluka (Buchs, Switzerland). Electrophoresis reagents, including coloured molecular weight markers and mini protean II apparatus, were from BIORAD (Hercules, CA). Mouse monoclonal antibodies specific for HSP70 (clone SPA 810) were from StressGen Biotechnology Corp. (Victoria, Canada), distributed by TEBU France.

Cell Culture Conditions

Isolation and culture of human desmodontal fibroblasts. Cells were obtained from periodontal ligament (PDL) explants dissected from the midroot of molar teeth extracted from healthy adults for nonperiodontal reasons. The PDL explant was mechanically removed by scraping the middle third of the root surface with a curette. The explants were put in 100 mm tissue culture plates in Minimum essential medium (MEM) supplemented with 5,000 IU/ml penicillin, 5,000 μg/ml streptomycin, 25 μg/ml fungizone, 2 mM L-Glutamine, and 10% heat-inactivated fetal calf serum (FCS). The plates were incubated at 37°C in a humidified incubator with 5% CO_2. After 2 weeks, the same culture medium containing less antibiotics and antifungics (100 IU/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone) was replaced every other day. Plates were microscopically examined for cell proliferation each day. When cell proliferation was evident, cells were dissociated with 0.25% trypsin and 0.05% EDTA. Desmodontal cells were subdivided into T-25 flasks for experimentation between the 2nd and 5th passages.

Mouse L929 fibroblasts. Mouse fibroblast cell line L929 (ATCC, Rockville, MD) was used throughout the experiments. The cells were routinely grown at 37°C in a 5% CO_2, 95% air atmosphere in Eagles Basic Medium containing 10% (V/V) fetal calf serum, 2 mM L-Glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. When cells reached confluence in T-75 flasks, they were dissociated with 0.25% trypsin and 0.05% EDTA in phosphate-buffered saline (PBS) and replated into T-25 plastic culture flasks.

He-Ne Laser Irradiation

Confluent cultures (cells cover at least 90% of the flask surface) were divided into control and laser-treated groups. The laser group was subjected to laser treatment (632.8 nm, 60 mW, Fradama SA, Geneva, Switzerland). The control group was subjected to sham treatment without laser light emission.

Laser irradiation was carried out through the wall of the flask and the culture medium. The focused laser beam (2 mm^2) was displaced by means of an automatic optical system (mirrors) to cover the total culture surface, and consequently, all the cells were equally irradiated by a quick scanning of all the culture surface. The laser power measured at the place of the sample with a photometer (UDT 371, Graseby optonics, Orlando, FL) was 52 mW. Each flask was irradiated for 12 or 24 min to obtain an energy density of 1.5 J/cm^2 or 3 J/cm^2, respectively.

A single application of the laser beam was performed in continuous mode on desmodontal fibroblasts at a density of 1.5 J/cm^2 ($n = 6$) or 3 J/cm^2 ($n = 6$). Control cells were lysed at 4°C after sham treatment in the same lysis buffer used under protein assays. Laser-irradiated desmodontal fibroblasts were lysed at 60, 120, or 180 min after the laser treatment. In the same manner, mouse L929 cells were also irradiated at 1.5 J/cm^2 ($n = 6$) or at 3 J/cm^2 ($n = 6$). They were lysed either directly at the end of the treatment (Time 0) or 30, 60, 120, 180, or 240 min after laser irradiation. Controls were lysed directly after sham treatment.

Protein Assays

After He-Ne Laser irradiations, all the cells were lysed at 4°C in a lysis buffer (composition: 150 mM NaCl, 1% NP-40, 50 mM Tris (pH 8,0) containing proteases inhibitors (Benzamidine 2 mM, phenylmethylsulfonyl fluoride 2 mM). Then, they were sonicated for 10 sec with a sonicator (model Sonifier B12 of Branson Sonic Power Co., Danbury, CT), set at 50% of its maximal capacity. Proteins were assayed on aliquots using the bicinchoninic acid method (Micro-BCA Kit from Pierce, Rockford, IL) using bovine serum albumin as standard. The optical density was measured at 562 nm using a spectrophotometer (Hewlett Packard Gmbh, Waldbronn, Germany).

Polyacrylamide Gel Electrophoresis and Western Blotting of HSP70

Gel electrophoresis (SDS-PAGE) was used to separate the proteins obtained after cell lysis according to their molecular weights. It was performed on a slab gel polyacrylamide (10%) and 0.1% sodium dodecyl sulfate under reducing conditions according to Laemmli [49] using a BioRad Mini-protean II apparatus. Equal amounts of proteins from cell lysates (40 μg) were loaded on electrophoresis gels. After migration, proteins were electrophoretically transferred to a nitrocellulose membrane in 0.2 M Tris/HCl buffer pH 9.2, at 150 mA for 3 hr at 4°C. This electric transfer allowed the detection of HSP70 on the nitrocellulose membrane using specific antibodies.

Completeness of the electric transfer was routinely controlled by staining polyacrylamide gels with 0.25% (W/V) Coomassie Blue R250 so-

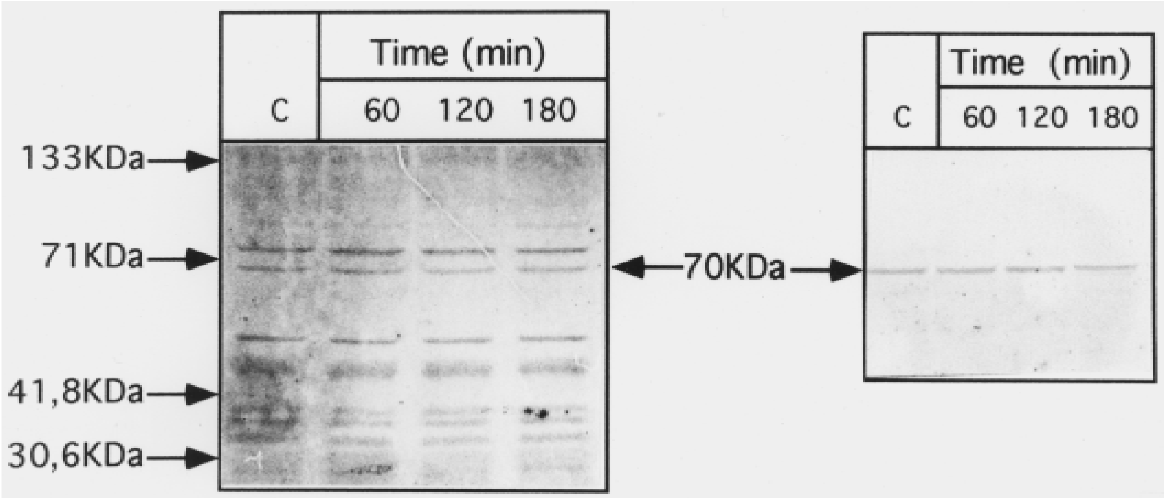

Fig. 1. Immunological staining of the heat-shock protein 70 of laser-irradiated (1.5 J/cm^2) human desmodontal fibroblasts. Confluent cultures were either sham-treated (c) or irradiated with He-Ne laser at 1.5 J/cm^2 and incubated for the indicated time. The position of the 70KDa stress protein is indicated (arrow) on the gel (Left) and on the nitrocellulose membrane (right). Molecular weight standards indicated on the electrophoresis gel in (KDa) are: β-galactosidase 133, bovine serum albumin 71, carbonic anhydrase 41.8, and soybean trypsin inhibitor 30.6.

lution (composed of ethanol/water/acetic acid (5 / 5 / 1 by volume). Destaining was performed in ethanol / acetic acid / water (2/ 3/ 35 by volume).

For HSP70 detection on nitrocellulose membranes, the membranes were blocked at room temperature for 45 min using 50 mM Tris / HCl buffer containing 150 mM NaCl and 3% (W/V) defatted bovine serum albumin (buffer A). Immunodetection was carried out using monoclonal antibodies directed against HSP70 (2 μg/ml) as primary antibodies. The incubation was performed overnight in buffer A plus 0.05% Tween 20 (V/V). After several washes, nitrocellulose membranes were incubated in buffer A containing alkaline-phosphatase-conjugated goat antimouse IgG. Alkaline phosphatase was revealed using 0.5 mM nitroblue tetrazolium and 0.5 mM 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M Tris/HCl pH 9.5 containing 0.1 M NaCl and 1 mM $MgCl_2$ for 10 min at room temperature.

Quantitative Analysis of Western Blots

Western blots were quantified using an image processing and analysis program (NIH Images, Version 1.49) (NIH, Bethesda, MD). This quantification allowed us to compare the level of HSP70 under the different treatment conditions. Results were expressed as percentage of control, which was considered as 100%.

Statistical Analysis

Data obtained from Western blots quantification were submitted to nonparametric analysis of variance using Kruskal and Wallis test. A value of $P < 0.05$ was considered statistically significant.

Cell Viability

Live/Dead Eukolight viability/cytotoxicity kit was used (Molecular Probes, Eugene, OR). This kit was used to distinguish living from dead cells in the same culture plate. The enzymatic conversion of the membrane permeant Calcein-AM gives rise to Calcein, which is retained within live cells producing an intense uniform green fluorescence in live cells. Ethidium homodimer enters cells with damaged membranes and undergoes a 40× enhancement of fluorescence on binding to nucleic acids, thereby producing a bright red fluorescence of the nucleus in dead cells. For each series of experiments, after laser irradiation of desmodontal as well as L929 fibroblasts, cell cultures were rinsed twice with PBS and incubated for 30 min at 37°C in PBS buffer containing 2 μM Calcein AM and 4 μM Ethidium homodimer (EthD-1). After labelling, the cells were rinsed with the PBS buffer. Calcein and EthD-1 were excited at 485 nm. Both positive and negative controls were performed. For negative control, cells were incubated in the culture medium, whereas for positive control, the cells were incubated with the same culture medium containing 6.4 mg/ml phenol for 30 min at 37°C. Cell labelling was carried out as described for laser-treated cells. Labelled cells were examined under an inverted fluorescence microscope.

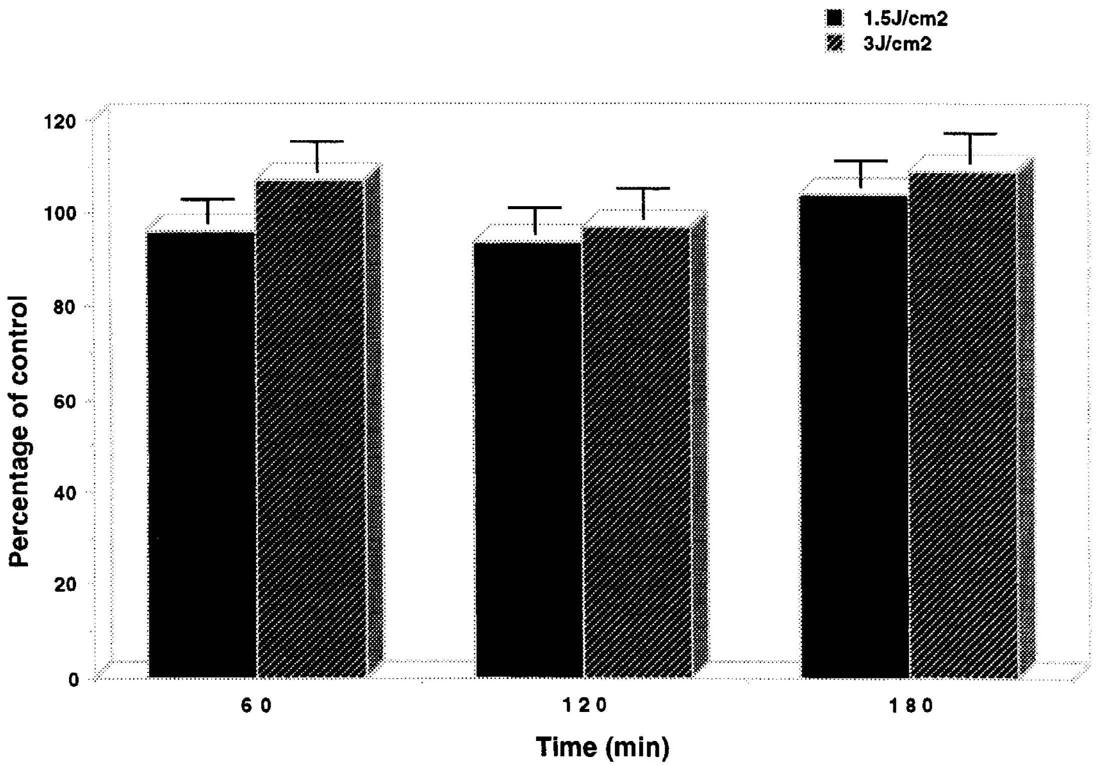

Fig. 2. Analysis of HSP70 level in human desmodontal fibroblasts by quantitative analysis of Western blots. Western blots were quantified with an image processing and analysis program. Time (min) indicates the incubation time elapsed after laser irradiation of cells. The results obtained after irradiating cells with 1.5 and 3 J/cm^2 are expressed as percentage of control ± S.D.

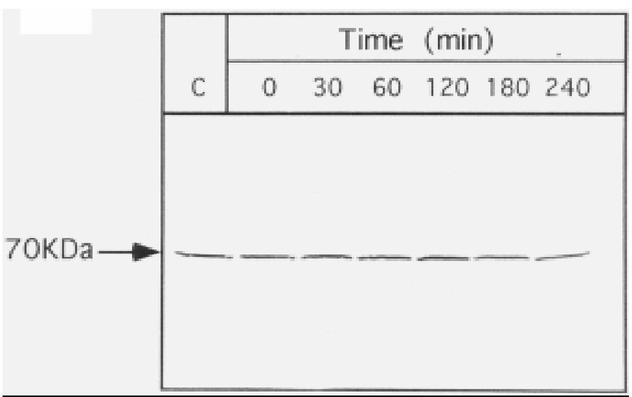

Fig. 3. HSP70 labelling of laser-irradiated (3 J/cm^2) mouse L929 fibroblasts. Cultures were sham-treated (C) or irradiated with He-Ne laser at 3 J/cm^2 and incubated for the indicated time. HSP70 position is indicated (arrow).

RESULTS

Kinetics of Heat-shock Protein 70 Synthesis in Desmodontal Fibroblasts

HSP70 synthesis kinetics was followed for 3 hr in desmodontal fibroblasts irradiated at 1.5 or 3 J/cm^2. As expected, after gel electrophoresis of proteins obtained from control and laser-irradiated desmodontal cells followed by the electric transfer, the monoclonal antibodies revealed a single band of 70 KDa in control and laser-irradiated cells on the nitrocellulose membranes corresponding to the HSP70 stress protein. The electrophoresis gel and the Western blot in Figure 1 show the results obtained from desmodontal cells irradiated at 1.5 J/cm^2. After irradiation, HSP70 synthesis was followed for 3 hr at 60-min intervals. No difference was observed between control cells and those lysed after laser irradiation. The level of HSP70 remained the same when the cells were incubated for 60, 120, or 180 min after laser treatment. Staining and destaining of the gels used for the electric transfer showed that the proteins were completely transferred onto the nitrocellulose membranes.

The kinetics of HSP70 synthesis in the cells subjected to a higher density (3 J/cm^2) of laser irradiation gave similar results (Fig. 2). After laser irradiation, HSP70 synthesis was studied on cell lysates for 3 hr. No difference was observed

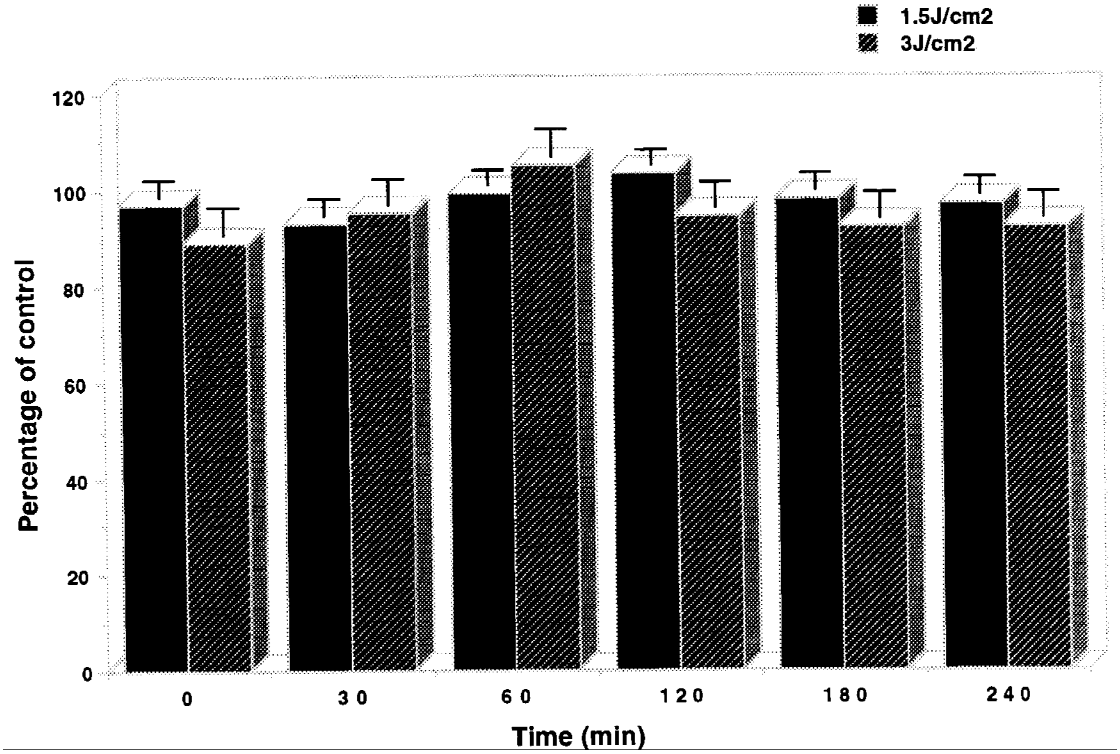

Fig. 4. Quantitative analysis of HSP70 in mouse L929 fibroblasts by image processing and analysis of Western blots. Western blots were quantified using an image processing and analysis program. Time (min) indicates the incubation time elapsed after cell irradiation with laser. The results obtained by irradiating cells at 1.5 and 3 J/cm^2 are plotted as percentage of control ± S.D.

between control and laser-irradiated cells lysed at 60, 120, or 180 min after the treatment. Quantitative analysis of the Western blots by image analysis (Fig. 2) and nonparametric analysis of variance confirmed the fact that HSP70 level at 60 min after laser treatment was not significantly different from that of control. In the same manner, no difference was observed at 120 or 180 min after laser irradiation. The Kruskal and Wallis test did not show any statistically significant difference between sham-treated cells and those irradiated at 1.5 or 3 J/cm^2. Results were expressed as percentage of control ± S.D.

Kinetics of Heat-shock Protein 70 Synthesis in Mouse L929 Fibroblasts

HSP70 level was followed over 4 hr in mouse L929 cells irradiated at 1.5 or 3 J/cm^2.

As shown with human desmodontal cells, HSP70 expression was not significantly different between control and laser-irradiated cells at 1.5 J/cm^2. The level of HSP70 was the same when the cells were lysed directly after laser irradiation (0) or incubated for 30, 60, 120, 180 or min after laser irradiation. The results obtained were almost the same when the cells were subjected to a higher irradiation density (3 J/cm^2). HSP70 expression was maintained at the same level, showing no difference between control and laser-irradiated cells at any incubation time. Figure 3 shows the effect of laser treatment at 3 J/cm^2 on HSP70 level in mouse L929 cells lysed either directly after irradiation (time 0) or after 30, 60, 120, 180, or 240 min of irradiation. Quantitative analysis of the level of HSP70 on Western blots after laser irradiation of cells with 1.5 or 3 J/cm^2 and nonparametric analysis of variance revealed no significant differences between control and laser-irradiated cells at any time after laser irradiation. In the same manner, the Kruskal and Wallis test did not show any statistically significant difference in the HSP70 level between control cells or those irradiated at 1.5 or 3 J/cm^2 (Fig. 4). Results were expressed as percentage of control ± S.D.

Cell Viability

The cell viability test was carried out for each series of experiments. Figure 5 (top) shows

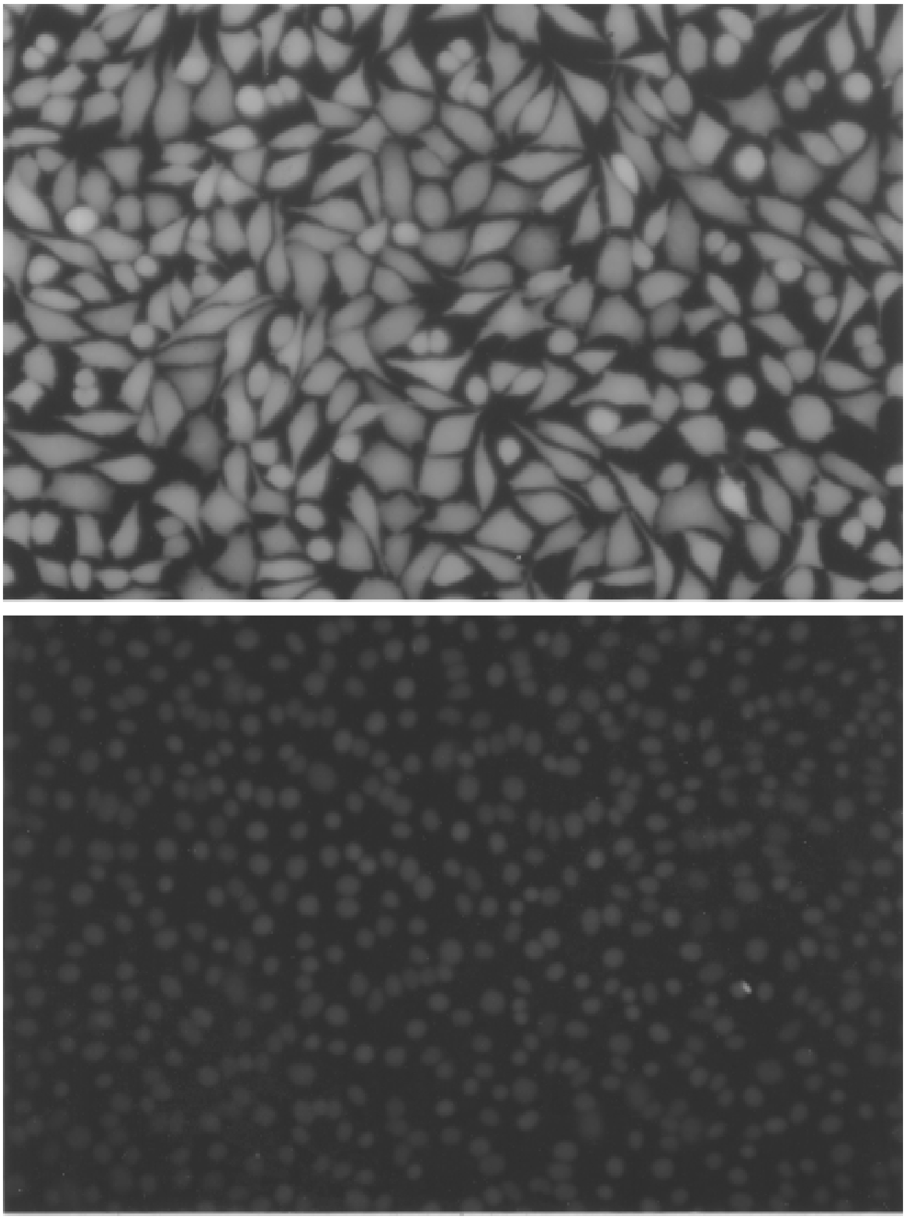

Fig. 5. Fibroblast viability test. L929 fibroblasts irradiated at 3 J/cm^2 and incubated for 3 hr after the treatment were examined for cell viability. Note the presence of living cells and the absence of dead ones in the laser-treated cells (top). Phenol-treated cells were used as positive control (bottom). They show dead cells only. (Original magnification × 20).

laser-treated L929 cells 3 hr after irradiation at 3 J/cm^2. All the cells were viable (green), and the results were the same in control and laser-treated cells at any time interval for both periodontal ligament cells and L929 fibroblasts. With the negative control, all cells were viable and appeared uniformly green, whereas with the positive control, phenol treatment was lethal to cells that showed red nuclei (Fig. 5, bottom).

DISCUSSION

The results of this study show that He-Ne laser irradiation does not stimulate HSP70 synthesis. This was shown using two different cell types from two different species: mouse L 929 fibroblast cell line and human desmodontal fibroblasts. Irradiations were performed at a density of 1.5 J/cm^2 or 3 J/cm^2. These densities are higher

than those used (1.2 J/cm^2) to study the effect of laser treatment on the growth dynamics and the ultrastructure of human gingival fibroblasts [50], or the transformation of fibroblasts into myofibroblasts [51], and twice higher than the density used in the prevention and treatment of chemotherapy and radiotherapy-induced mucositis [16, 52].

Previous studies on organ-cultured human skin [48] and normal fibroblasts (W138VAB) [53] have shown by immunofluorescence that HSP70 is highly induced after heat shock at 45°C and attains a maximum after incubation at 37°C for 2 hr. In the present work, HSP70 level was studied in desmodontal cells over a 3-hr period.

In agreement with the literature, in control cells, the level of HSP70 was relatively low. This fact is in agreement with the function of these proteins under normal growth conditions, since they stabilize unfolded protein precursors as well as newly translocated proteins into organelles such as the mitochondria and the endoplasmic reticulum. But following heat shock, or other forms of stress, their synthesis increases dramatically to ensure survival under these stressful conditions, which, if left unchecked, lead to irreversible cell damage and ultimately cell death. In our study, when the cells were irradiated at 1.5 J/cm^2, no stimulation was observed even when the cells were incubated for 3 hr after irradiation. The results were similar when the cells were irradiated at a density of 3 J/cm^2. This indicates that He-Ne laser irradiation is not a stressful treatment to these cells at the densities used in this study.

The high metabolic activity of mouse L929 fibroblasts explains the level of HSP70 in these cells, which is higher than that of human desmodontal fibroblasts. After irradiation of mouse L929 fibroblasts at 1.5 or 3 J/cm^2, the expression of HSP70 was stable even when the irradiated cells were incubated for 4 hr after irradiation.

In our experiments, the absence of stimulation in HSP70 synthesis can not be due to incomplete transfer of proteins from the electrophoresis gels on to the nitrocellulose membranes. In all experiments the completeness of the electric transfer was controlled by staining and destaining of the gels used for the electric transfer, which were always negative. Cell viability was also controlled in each experiment by the viability toxicity method. This two-colour fluorescence cell viability assay is based on the simultaneous determination of live and dead cells with two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity. This method has shown that all cells were viable, and no alteration due to the irradiation was observed. These results indicate clearly that laser irradiation of cells does not affect their viability or stimulate stress proteins synthesis. Although, HSP70 is the major stress-inducible form of the HSP families [39, 40, 54], it is not the only stress inducible one. HSP90 family proteins are highly expressed under normal growth conditions, and they can be further induced by heat shock or other forms of stress [43]. However, in some experiments, we studied the effects of laser irradiation on HSP90 synthesis by Western blotting using monoclonal antibodies to HSP90. The results obtained did not show any stimulation of HSP90 protein synthesis (not shown).

Taken together, these results show that He-Ne laser irradiation is not a stressful treatment to cells subjected directly to this treatment. Although some clinical studies showed no significant side effects of He-Ne irradiation, this is the first work to demonstrate the absence of side effects at the cellular level. Our results indicate that the He-Ne laser treatment might continue to be used in clinic in the treatment of chemotherapy-induced mucositis preventively as well as curatively with no considerable risk of side effects at the cellular level.

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